Development of a Novel Enzyme-free Approach to Determine *N'*-Nitrosonornicotine (NNN) in Human Urine

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Introduction

N'-nitrosonornicotine (NNN) is a tobacco-specific *N*-nitrosamine that is formed during the curing process and is classified as a Group 1 carcinogen by the International Agency for Research on Cancer. NNN is widely used for biomonitoring tobacco exposure via smoke or smokeless. It is metabolized to NNN-*N*-glucuronide and is present in the human body in the free and glucuronide forms. Therefore, the standard approach is to measure total NNN after enzymatic deconjugation of the glucuronide by β-glucuronidases. However, there are well-known challenges with measuring NNN in urine:

- β-glucuronidases are more efficient in deconjugating *O*-glucuronides than *N*-glucuronides.
- Nornicotine converts to NNN in acidic (pH<6) conditions in the presence of nitrites and nitrates, which might happen with urine samples to be analyzed and therefore lead to overestimation of NNN concentration.

Our aim is to establish a robust high-throughput method to measure total NNN in human urine.

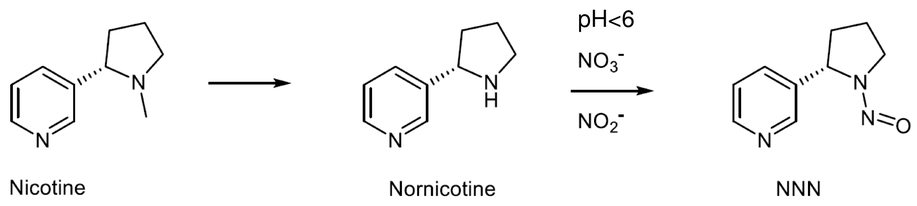

Figure 1. NNN formation from nornicotine in acidic conditions in the presence of nitrites and nitrates.

Method

Figure 2. Sample extraction procedure from human urine

- STDs are prepared in water
- QCs are prepared in non-smoker urine
- Assay volume: 1 mL urine
- Sample preparion on 96-deep-well plate
- Incubations were carried out in Eppendorf Thermomixer
- SPE: Waters Oasis MCX plate

Table 1. Chromatographic conditions and MS/MS parameters

Chromatographic conditions	
UHPLC	Waters ACQUITY UPLC I-Class
Analytical column	ACE Excel C18-PFP 150 × 2.1 mm, 1.7 µm
Mobile phase A **Mobile phase B**	Acetic acid in water / Acetonitrile / Water 10:5:85 (v/v/v) Acetic acid in water / Methanol / Water 10:70:20 (v/v/v)
Flow rate **Column temperature** **Injection volume**	0.5 mL/min 50 °C 20 µL
Total run time	8.0 min
MS/MS conditions	
Mass spectrometer	SCIEX Triple Quad 6500+
Source / Polarity	ESI / Positive
Followed MRM transitions	NNN: m/z 178.1 → m/z 148.1 NNN-$^{15}N_2$-^{13}C (IS): m/z 181.1 → m/z 150.1

Chromatograms

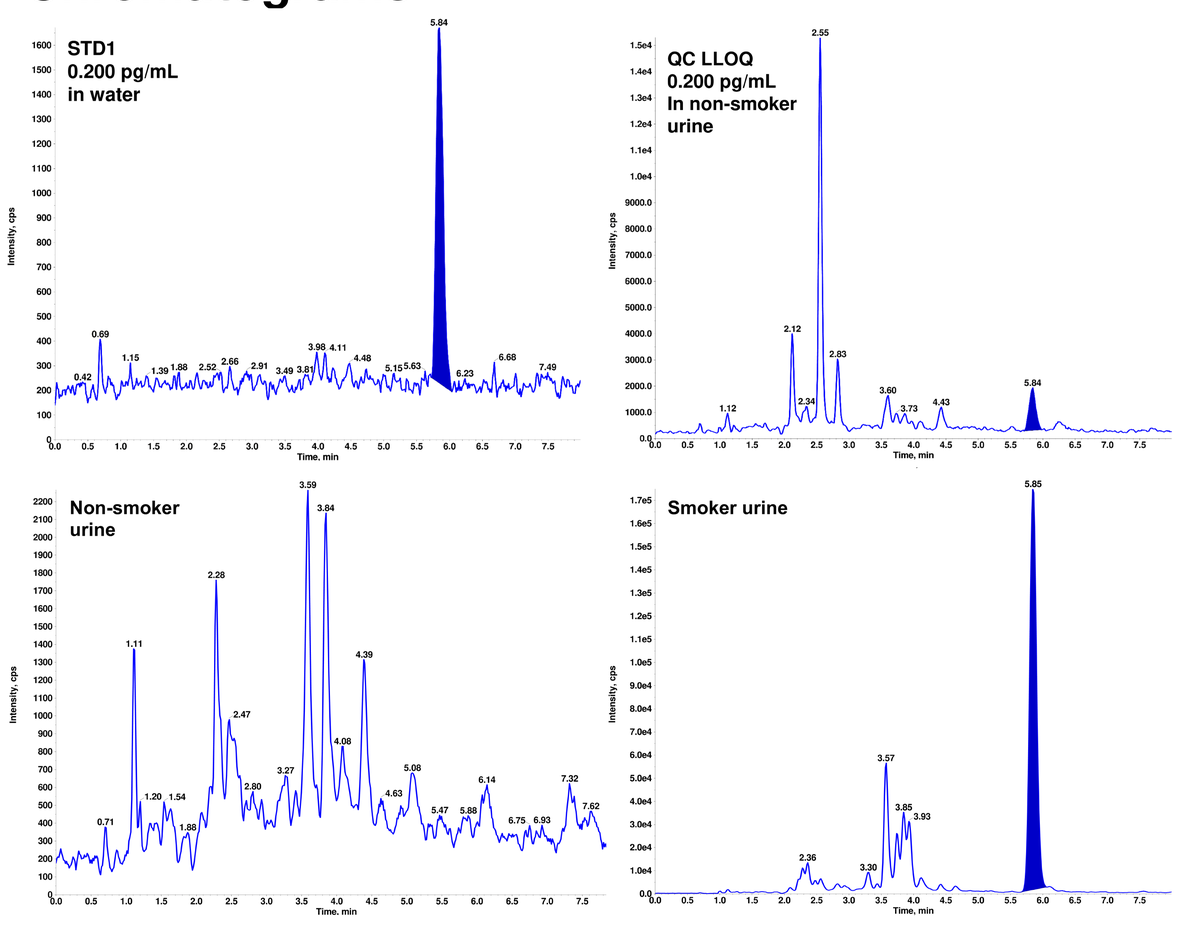

Figure 3. Typical chromatograms of STD1 in Millipore water, QC LLOQ in non-smoker urine, non-smoker urine and a smoker urine pool.

Results

- NNN concentrations were measured in a smoker urine pool and expressed in fold change, i. e. the free NNN concentration after hydrolysis divided by the initial (t=0) free NNN concentration.
- Enzymatic deconjugation: 5 β-glucuronidases were investigated from different sources in order to check whether there are efficiency differences. The reactions were carried out at 55 °C.
- Chemical deconjugation: NaOH was used in order to avoid conversion of nornicotine under acidic conditions. 1M NaOH was shown to fully deconjugate nicotine-*N*-glucuronide in 35 min at 75 °C[1]. In an initial experiment these parameters were examined, however 35 min was not long enough for complete deglucuronidation of NNN-*N*-glucuronide.

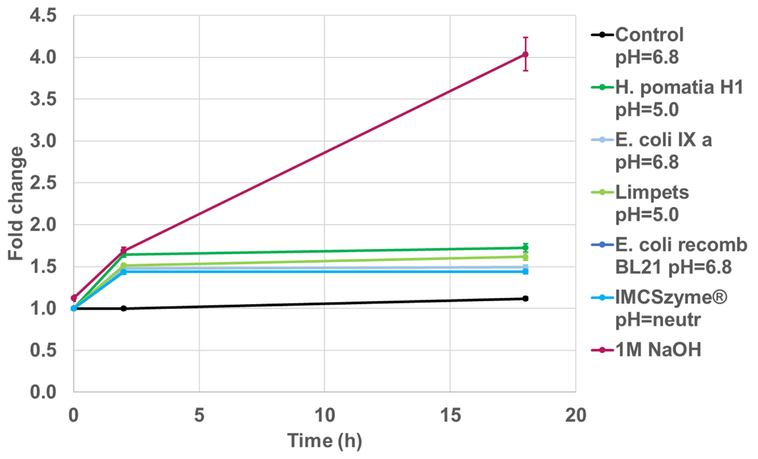

Figure 4. NNN fold changes measured in a smoker urine pool after enzymatic or chemical deconjugation.
n=3 replicates per condition, error bars represent ± SD.

The results show that 1M NaOH was the most efficient to deconjugate NNN-*N*-glucuronide. The enzymes showed a much lower efficiency.

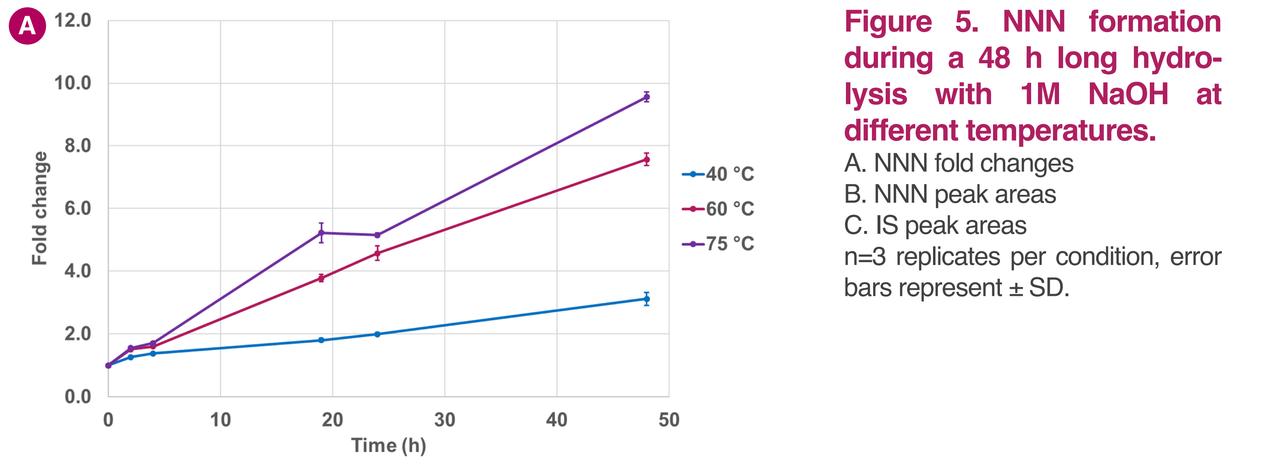

Figure 5. NNN formation during a 48 h long hydrolysis with 1M NaOH at different temperatures.
A. NNN fold changes
B. NNN peak areas
C. IS peak areas
n=3 replicates per condition, error bars represent ± SD.

The IS peak areas indicate that NNN starts to degrade during hydrolysis with 1M NaOH at 75 °C from 19 h on. Therefore most probably there is a simaultaneous free NNN formation from deglucuronidation and degradation due to the high temperature. The reactions incubated at 40 and 60 °C showed no degradation within 48 h.

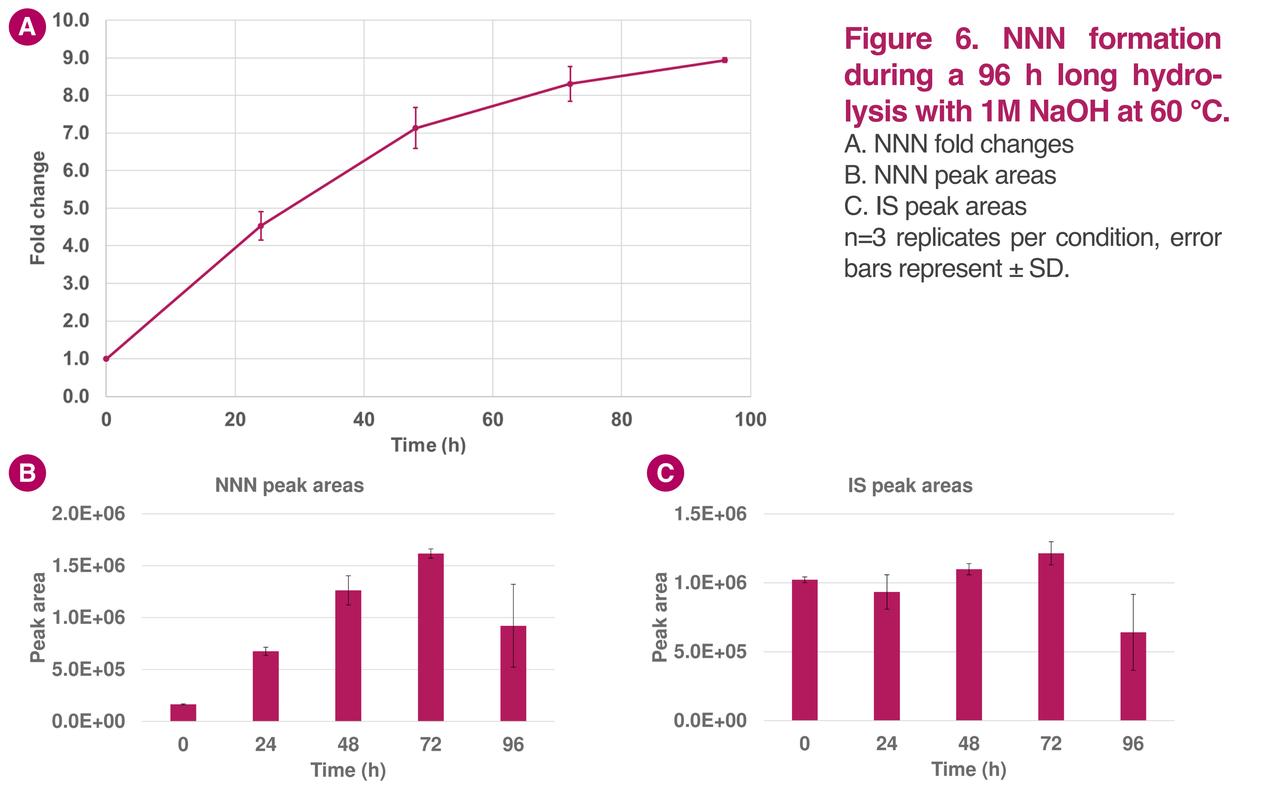

Figure 6. NNN formation during a 96 h long hydrolysis with 1M NaOH at 60 °C.
A. NNN fold changes
B. NNN peak areas
C. IS peak areas
n=3 replicates per condition, error bars represent ± SD.

The IS peak areas indicate that NNN starts to degrade during hydrolysis with 1M NaOH at 60 °C between 72-96 h. The optimum hydrolysis time is 72 h at 60 °C with 1 M NaOH.

Conclusions

- NaOH proved to be the most efficient way for deconjugating NNN-*N*-glucuronide. All tested enzymes were not capable of complete deglucuronidation.
- The assay is able to measure NNN from 1 mL urine in the concentration range of 0.200-40.0 pg/mL.
- Urine samples for NNN measurement need to be buffered right after collection to avoid artefactual formation of NNN from nornicotine.
- Currently the hydrolysis paramaters are being optimized in order to establish a robust high-throuhput NNN assay in human urine.

[1]Benowitz NL, Jacob P 3rd, Fong I, Gupta S. Nicotine metabolic profile in man: comparison of cigarette smoking and transdermal nicotine. J Pharmacol Exp Ther. 1994 Jan;268(1):296-303. PMID: 8301571.